EXHIBIT Q

Alkylation of Rat Liver DNA by Dimethylnitrosamine: Effect of Dosage on O^6-Methylguanine Levels[1,2]

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ABSTRACT—Alkylation of liver DNA was studied following administration to Sprague-Dawley rats of doses of dimethylnitrosamine (DMN) varying from 0.25 to 20 mg/kg body weight. Measurements were made of the amounts of O^6-methylguanine and 7-methylguanine present in liver DNA at 4 and 24 hours after treatment with the carcinogen. There was a linear relationship between 7-methylguanine levels and dose of the nitrosamine at both of these times. In contrast, the corresponding levels of O^6-methylguanine were not directly proportional to dosage but were less than expected, particularly at low doses below 2.5 mg/kg. This discrepancy was significant at 4 hours, but was even more marked at 24 hours. Only doses above 4 mg/kg at the 4-hour time point gave rise to a 0.11 ratio of alkylation of guanine at the O^6- position to that at the 7- position. This ratio was that expected for the initial interaction of the alkylating species derived from DMN with DNA. Evidence was obtained to support the hypothesis that these results were due to an enzymatic removal of O^6-methylguanine from liver DNA, which occurred much more efficiently at lower initial levels of alkylation. Repeated daily injections of DMN up to 11 days also gave rise to O^6-methylguanine levels that were not proportional to dosage but were relatively greater at higher dose levels. The significance of these findings in the induction of liver cancer by feeding or repeated injection of DMN was explored.—J Natl Cancer Inst 58: 681–687, 1977.

It is generally accepted that DMN exerts its carcinogenic action by means of its metabolic conversion into a highly reactive methylating agent *(1–5)*. This agent reacts with many cellular constituents, including DNA. Although by no means proven, it has been assumed as a working hypothesis that the alkylation of DNA is the basis for the initiation of neoplastic growth *(1–9)*. The major product of the reaction of DMN with DNA is 7-methylguanine but this product is probably not responsible for tumor induction, since several studies have shown no correlation between the production of 7-alkylguanine and the carcinogenicity of a number of alkylating agents and N-nitroso compounds *(6–9)*. Furthermore, 7-alkylguanine does not lead to incorporation of incorrect nucleotides by nucleic acid polymerases in vitro and may not be a promutagenic product in vivo *(3, 10–13)*. For these reasons more attention has been given recently to the other products of the alkylation of DNA. In particular, the formation and properties of O^6-alkylguanine have been studied. This product has been implicated in mutagenesis *(3, 11–13)* and does promote misincorporation by nucleic acid polymerases *(14)*. In a number of recent investigations the formation and persistence of this product in DNA of various organs after administration of N-nitroso carcinogens has been shown to correlate with the incidence of tumors *(9, 15–19)*.

After administration of large single doses of DMN to rats, the ability of the kidney to remove O^6-alkylguanine from its DNA was much less than that of the liver *(2, 9, 18)*. Such treatment does produce kidney tumors *(1, 6)*, but liver tumors are not obtained after single doses of DMN to normal adult rats. However, repeated daily treatment or feeding of DMN to rats results in a high incidence of liver cancer *(1, 20, 21)*. Therefore, in attempts to discover whether the correlation between O^6-alkylguanine levels in DNA and tumor production also exists in the induction of liver tumors by the nitrosamine, it was necessary to measure the formation of this base and its persistence in the liver over a 24-hour period. This paper describes the effect of various doses of DMN on O^6-methylguanine levels under these conditions. It was found that the levels were not directly related to the dose but were disproportionately greater after higher doses. This difference was also seen after repeated daily treatments with DMN.

MATERIALS AND METHODS

Chemicals.—[^{14}C]DMN (5.185 mCi/mmole) was purchased from New England Nuclear, Boston, Massachusetts. In certain experiments it was diluted with unlabeled DMN, which was obtained from Eastman-Kodak, Rochester, New York, and redistilled before use. *N*-[^{14}C]Methyl-*N*-nitrosourea was synthesized from *N*-[^{14}C]methylurea (4.8 mCi/mmole; New England Nuclear) as previously described in *(6, 9)*. O^6-Methylguanine was synthesized by the reaction of 2-amino-6-chloropurine with sodium methoxide *(22)*. All other biochemicals were purchased from Sigma Chemical Company, St. Louis, Missouri.

Treatment of animals.—Adult female Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 160–170 g were used. DMN was administered by ip injection of a solution in 0.9% (wt/vol) NaCl between 10:00 and 11:00 a.m. The concentration of [^{14}C]DMN in the solution for injection was adjusted so that a total volume between 0.3 and 0.6 ml was administered. All solutions of DMN were protected from light and stored at −20° C. Rats given repeated injections of DMN were allowed access to food at all times and were weighed daily. Those treated with 0.5 mg/kg body weight gained weight at the same rate as untreated controls. Those given 5 mg/kg body weight did not gain weight and in some cases lost up to 15 g over the 11-day period.

N-[^{14}C]Methyl-*N*-nitrosourea was dissolved in 5 mM sodium citrate buffer containing 0.9% (wt/vol) NaCl and administered by iv injection. Rats were killed by cervical dislocation and the livers rapidly removed, frozen in liquid N_2, and stored at −60° C until required.

Abbreviation used: DMN = dimethylnitrosamine.

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Alkylation of DNA in vitro.—Calf thymus DNA was alkylated in vitro by incubation of a solution containing 10 mg DNA, 100 mM sodium phosphate buffer (pH 7.5), and 0.1 mM *N*-[^{14}C]methyl-*N*-nitrosourea for 1 hour at 37° C. The DNA was then precipitated by the addition of three volumes of cold 2-ethoxyethanol, washed four times by dissolving in 3 M sodium acetate (pH 5.5), and reprecipitating with 2-ethoxyethanol and hydrolyzed as described below.

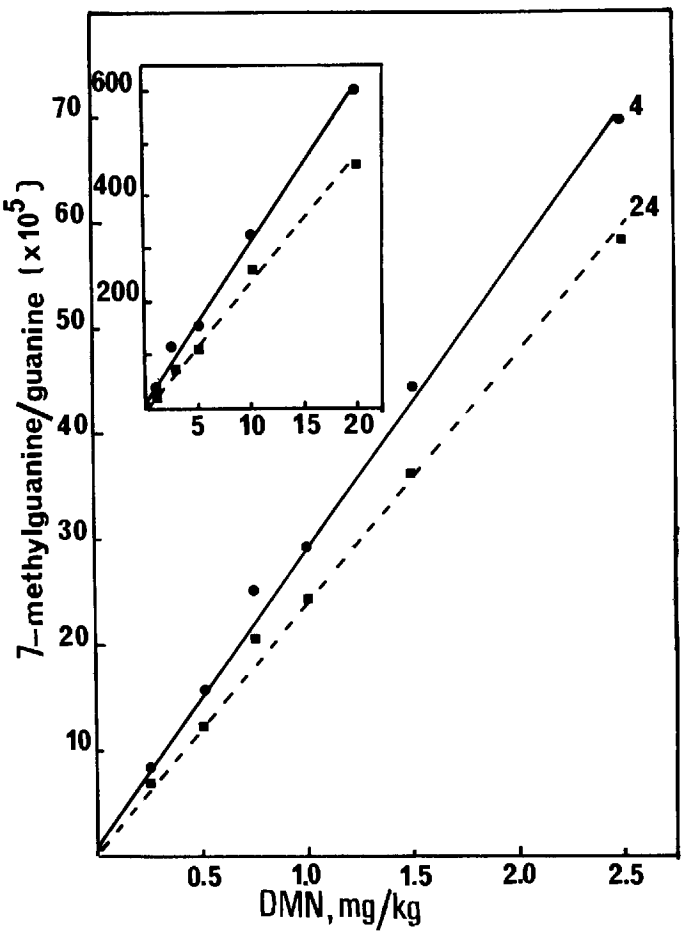
Analysis of DNA.—DNA was prepared from the frozen liver samples as previously described *(9, 18)* and hydrolyzed in 0.1 N HCl at 37° C for 20 hours or at 70° C for 30 minutes as described by Lawley and Thatcher *(23)*. This procedure leads to the release of all of the purines as free bases. After hydrolysis, unlabeled marker compounds of the methylated purines being studied were added and the sample applied to a column (70×1.6 cm) of Sephadex G10. The column was then eluted with 0.05 M ammonium formate, 0.02% sodium azide (pH 6.8) at a flow rate of 40 ml per hour. The absorbance at 254 nm of the eluate was monitored with a Uvicord II spectrophotometer and fractions of 5 ml were collected. Good separations of guanine, adenine, 3-methyladenine, 3-methylguanine, 7-methylguanine, and O^6-methylguanine were obtained as previously reported *(9, 17–19, 24)*. The amounts of guanine and adenine present were determined by measurement of the absorbance at 260 nm of the relevant fractions. (It was assumed that the molar extinction coefficient of guanine was 7,000 liters/mole/cm and of adenine was 13,300 liters/mole/cm in the ammonium formate buffer at pH 6.8.)

Radioactivity in each fraction was determined by addition of 10 ml of Formula-947 LSC Cocktail (New England Nuclear) and counting in a Beckman LS-350 liquid scintillation counter. The efficiency of counting, determined by the addition of an internal standard, was around 65%. The amounts of the methylated bases that had been produced by treatment with DMN were then calculated from the radioactivity in the fractions, which corresponded to authentic marker compounds. Sufficient DNA was analyzed to yield enough radioactivity in the O^6-methylguanine peak for this to be counted with 95% confidence at ±5% error. The levels of alkylated derivatives were expressed as a function of the amount of the parent base present in DNA. (The degree of alkylation would, therefore, be underestimated if new DNA synthesis occurred after alkylation had taken place, since this would increase the total amount of purine bases. However, this is unlikely to be important in the adult rat liver during the relatively short period of time of most of the experiments described in this paper.)

In order to ensure that errors were not introduced due to the incomplete hydrolysis of the DNA or loss of guanine due to the insolubility of this base at neutral pH, the adenine:guanine ratio of each hydrolysate was calculated and was always found to be within the range 1.55–1.70. All of the results shown represent the mean of at least two estimations which did not differ by more than 10%. Results for livers from 2 or more rats were combined for each estimation. The greatest source of error in these estimations is probably in the determination of guanine as described above. Hence the ratio of O^6-methylguanine:7-methylguanine—which does not depend on knowledge of the guanine present but only on the radioactivity in different fractions from the same column eluate—is probably more accurate than the individual measurements of the alkylated bases (text-fig. 3; tables 1, 2).

RESULTS

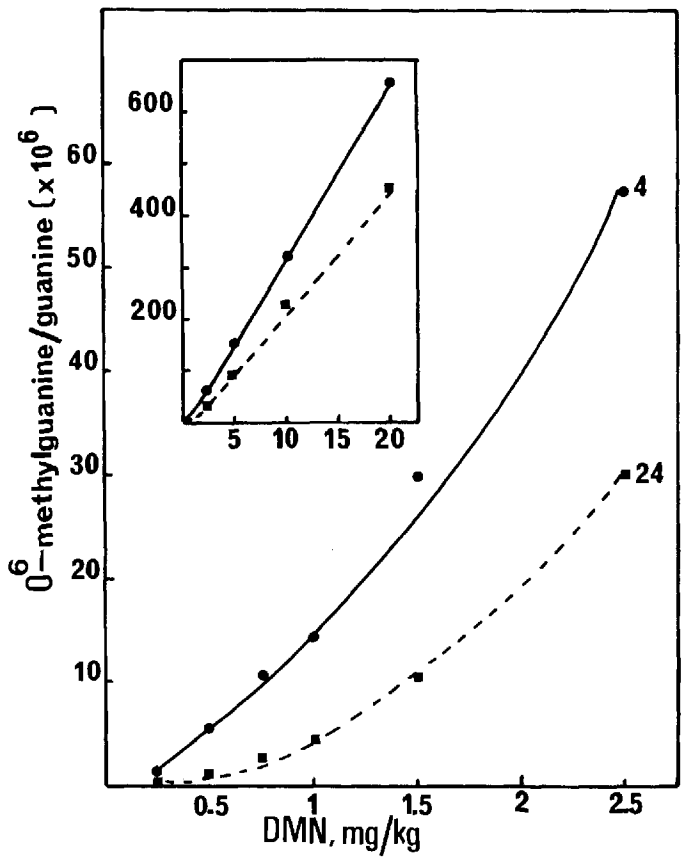
The amounts of 7-methylguanine in rat liver DNA at 4 and 24 hours after administration of DMN are shown in text-figure 1. The amount of 7-methylguanine present at either time was directly proportional to the dose of DMN administered over the entire range from 0.25 to 20 mg/kg body weight. Similar results were previously reported by Craddock *(25)*, although the lowest dosage in those experiments was 1 mg/kg body weight. The rate of metabolism of DMN in the rat is such that all of the administered dose is metabolized in 4 hours even at the highest dose employed *(26, 27)*. The values at 4 hours (about 0.031% of the guanines present in the DNA were



TEXT-FIGURE 1.—7-Methylguanine levels in rat liver DNA after administration of various doses of DMN. Results are shown for the amounts of 7-methylguanine present at 4 hours (●——●) and 24 hours (■- - -■) after injection of DMN. Values for doses of DMN varying from 0.25 to 2.5 mg/kg body weight are shown in the main figure and for doses of 1–20 mg/kg in the insert. 7-Methylguanine levels are expressed as moles/10^5 moles of guanine.

methylated/mg DMN/kg body weight), therefore, represent almost the maximal level of alkylation but with a slight underestimation, since 7-methylguanine was probably being lost from the liver DNA at the same time as it was being produced. This loss would not have been very great since the decline in 7-methylguanine levels between 4 and 24 hours after treatment with DMN occurred at a similar rate for all doses, with a half-life of about 70 hours. This half-life was in reasonable agreement with previous studies over a longer time period *(9, 17–19, 24)*. As discussed above, although there is convincing evidence that the formation of 7-methylguanine is not the critical product in the development of tumors, it provides a useful measurement of the generation of the alkylating intermediate and of a control for the studies of O^6-methylguanine.

Text-figure 2 shows similar data for O^6-methylguanine formation as a function of dose of DMN. Although after doses of more than 5 mg/kg (*see* insert, text-fig. 2) O^6-methylguanine levels were proportional to dose, at lower doses this was not the case and even 4 hours after DMN administration O^6-methylguanine levels were less than expected. By 24 hours after treatment with the carcinogen this phenomenon was even more apparent (text-fig. 2).



TEXT-FIGURE 2.—O^6-Methylguanine levels in rat liver DNA after administration of various doses of DMN. Results are shown for the amount of O^6-methylguanine present at 4 hours (●——●) and 24 hours (■- - -■) after injection of DMN. The main figure shows results for DMN doses of 0.25–2.5 mg/kg body weight; *the insert* shows results for doses of 1.5–20 mg/kg body weight. O^6-Methylguanine levels are expressed as moles/10^6 moles of guanine.

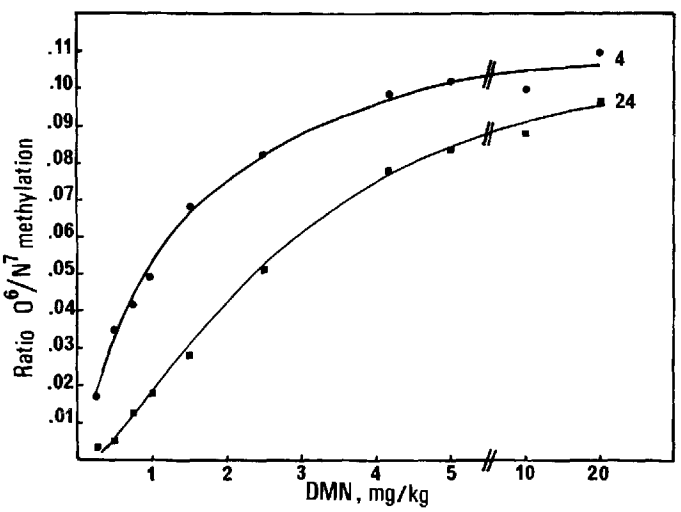
There are two possible explanations for the data given in text-figure 2 showing that O^6-methylguanine levels were not directly proportional to the dose of DMN. One possibility is that the initial reaction of the alkylating species derived from DMN with the O^6-position of guanine is relatively higher at higher doses of DMN; the other possibility is that the O^6-methylguanine is lost from the DNA and is lost at a more rapid rate after the lower doses. The latter possibility appears to be correct when the observations over the 4–24-hour period are considered. It can be seen from text-figure 2 that, after all doses of DMN, O^6-methylguanine levels fell between 4 and 24 hours but a greater proportion of the initial amount was lost after the lower doses. The apparent half-life of O^6-methylguanine as calculated between these two time points, therefore, varied from about 6 hours at the lowest dose to about 30–40 hours after the highest doses. Values for the half-life of O^6-alkylguanine loss from rat liver DNA, which have been calculated over longer periods of time up to 96 hours, varied from 13 to 26 hours [reviewed in *(9)*]; the greater values were obtained after the higher doses of alkylating agents.

Table 1 and text-figure 3 give further evidence supporting the hypothesis that the lower levels of O^6-methylguanine produced after doses of DMN below 2.5 mg/kg are due to preferential loss of this product. Text-figure 3 shows the ratio of O^6-methylguanine:7-methyl-

TABLE 1.—*Alkylation of rat liver DNA by* N-*methyl*-N-*nitrosourea*

N-[^{14}C]Methyl-*N*-nitrosourea treatment	Hours of exposure	Methylated bases moles/10^6 moles guanine		Ratio of O^6-methylguanine:7-methylguanine
		O^6-Methylguanine	7-Methylguanine	
19.2 mg/kg	1	24.2	310	0.078
19.2 mg/kg	2	19.7	326	0.060
19.2 mg/kg	7	11.2	304	0.037
0.1 mM in vitro[a]	1	59.4	552	0.108

[a] Calf thymus DNA was alkylated in vitro by incubation at 37° C with *N*-[^{14}C]methyl-*N*-nitrosourea.



TEXT-FIGURE 3.—Effect of dose of DMN on ratio of O^6-methylguanine to 7-methylguanine in rat liver DNA. Results are shown for estimations 4 hours (●——●) and 24 hours (■——■) after injection of DMN.

guanine found in the liver DNA as a function of dose of DMN. It is known that the reaction of *N*-methyl-*N*-nitrosourea with DNA in vitro produces these products in the ratio of about 0.11 *(3, 9;* table 1*)*. Since DMN is metabolized to an alkylating species thought to be similar to that generated chemically by *N*-methyl-*N*-nitrosourea *(2, 4, 5)*, it would be expected that the initial ratio of attack at the O^6- as compared to that at the 7-position of guanine would be the same for both agents. This was indeed the case for the higher doses of DMN in the liver [text-fig. 3; *(9, 18)*] and kidney *(9, 18)*. However, as shown in text-figure 3, even at only 4 hours after treatment this ratio was considerably less than expected for doses of DMN of less than 4 mg/kg body weight, and became progressively less as the dosage of DMN was decreased. By 24 hours this ratio was reduced for all doses, since the rate of loss of O^6-methylguanine from the DNA was greater than the rate of loss of 7-methylguanine.

It therefore appears that at least part of the reduction in O^6-methylguanine levels found after low doses of DMN was due to the more rapid removal of this product after the lower doses. However, on the basis of these data we cannot entirely rule out the possibility that at low doses of DMN there is for some reason a reduced attack of the alkylating species on the O^6-position of guanine in DNA. Since DMN must be metabolized by the liver to the active alkylating species it is difficult to design a suitable experiment to prove that the initial attack at the O^6-position does occur at the expected value of 0.11 × that at the 7-position. Therefore, this experiment was carried out with *N*-methyl-*N*-nitrosourea. This nitrosamide has a half-life of only a few minutes in the bloodstream *(28)* and is known to decompose rapidly at physiological pH to yield an alkylating species *(1, 4, 5)*.

Rats were thus given a dose of *N*-[^{14}C]methyl-*N*-nitrosourea (19.2 mg/kg body weight) which produced an amount of O^6-methylguanine similar to that produced by 1 mg DMN/kg body weight and killed at 1 hour, 2 hours, and 7 hours later. The amounts of alkylated guanines present in the DNA are shown in table 1, which also has data for calf thymus DNA alkylated in vitro by reaction with the nitrosamide. The maximal value of O^6-methylguanine found was at 1 hour after treatment with the nitrosamide and was more than twice that found at 7 hours. There was little change in the 7-methylguanine levels over this period. At the 1 hour time point the O^6-:7-methylguanine ratio was 0.078; this ratio fell to 0.037 at 7 hours. By 4 hours after DMN administration the ratio was 0.049. These results indicate that 1 hour after the nitrosamide was given, a higher initial level of alkylation at the O^6-position of guanine was achieved, and that this purine was then rapidly lost from the DNA. However, as shown in table 1, after reaction of calf thymus DNA with the nitrosamide in vitro the ratio of O^6-:7-methylation was 0.108, considerably in excess of that seen in vivo even at the shortest time. This ratio agreed well with that obtained by others using different concentrations of *N*-methyl-*N*-nitrosourea *(3, 9)*. It remains possible, therefore, that the initial reaction of the alkylating species with the O^6-position of guanine in liver DNA in vivo is somewhat less than in vitro.

TABLE 2.—*Alkylation of rat liver DNA by repeated daily doses of DMN*

Dose of DMN mg/kg/day	Days[a]	Methylated bases moles/10^6 moles guanine		Ratio of O^6-methylguanine:7-methylguanine
		O^6-Methylguanine	7-Methylguanine	
0.5	1	1.2	124	0.010
0.5	7	6.7	458	0.015
0.5	9	7.3	572	0.013
0.5	11	8.4	560	0.015
5.0	1	99	1,176	0.084
5.0	7	488	4,576	0.107
5.0	9	442	4,920	0.090
5.0	11	454	4,730	0.096

[a] The rats were killed 24 hr after the final injection.

The results described above indicate that there was an enzymatic pathway leading to the loss of O^6-methylguanine from liver DNA [since this purine was stable in DNA incubated in vitro *(3, 9, 16)*], and that this pathway was more efficient after lower doses of DMN. These results apply to the 24-hour period following a single administration of DMN. In order to determine whether this situation also applied following repeated treatment with the carcinogen, rats were given daily injections of DMN up to 11 days and killed 24 hours after the last injection. The results are shown in table 2 for rats given daily doses of either 0.5 or 5 mg/kg and killed 1 day, 7 days, 9 days, and 11 days after the first injection. For the rats receiving the higher doses of DMN there was little difference between the amounts of alkylated guanines present at 7, 9, and 11 days, which indicated that a stable plateau balancing production and removal had been reached. The levels of alkylated bases present at that time were about 5 times those present after 1 day; the O^6-:7-methylguanine ratio was about 0.09–0.10. After the lower dose of 0.5 mg/kg/day, the corresponding levels of 7-methylguanine present in the liver DNA were between 9 and 12% of those produced by 5 mg/kg and were also about 5 times those produced after 1 day. The O^6-methylguanine levels were only about 1.3–1.9% of those produced by the dose that was 10 times greater. Therefore, it appeared that the preferential loss of O^6-methylguanine that occurred after a low single dose of DMN was also seen after repeated daily treatment with these doses. This phenomenon was reflected in the O^6-:7-methylation ratio of guanine in the liver DNA, which was 0.015 after 11 daily doses of 0.5 mg DMN/kg and 0.096 after 5 mg/kg.

DISCUSSION

At least 10 sites of attack on nucleic acids by alkylating agents have been detected in recent studies *(3, 5, 29)*. The formation of some of these products was shown not to correlate with carcinogenesis when agents with differing carcinogenic potency and ability to form these products were compared *(5–9)*. These included the major product of the reaction, 7-alkylguanine, as well as the

minor products, 3-alkyladenine, 1-alkyladenine, 3-alkylcytosine, and 3-alkylthymine. If alkylation of DNA is important in carcinogenesis, the critical stimulus apparently must be produced by one of the other products of the alkylation reactions. These include alkylation of phosphates *(29–32)* forming phosphotriesters which in DNA are chemically stable *(33)*, 3-alkylguanine *(34)*, O^4-alkylthymine *(35)*, 7-alkyladenine *(29)*, and O^6-alkylguanine *(3, 9, 12, 15–19)*. The most likely of these products to be responsible for carcinogenesis is O^6-alkylguanine. The biologic effects of the formation of phosphotriesters in DNA are unclear, and the other alkylated bases are produced in much smaller amounts than O^6-alkylguanine. The production of O^6-alkylguanine has been implicated in mutagenesis *(3, 11–13)*; this product causes misincorporation by bacterial nucleic acid polymerase *(14)*. A number of studies have measured the formation and persistence of O^6-alkylguanine in target tissues after treatment with N-nitroso carcinogens. These investigations, reviewed recently in *(36)*, have revealed that organs which readily develop tumors after a single dose of carcinogen *(17)* are much less active in removing O^6-alkylguanine from their DNA than the liver *(9, 15–18, 37)*. Liver tumors are not produced by single doses of DMN or *N*-methyl-*N*-nitrosourea to adult rats unless preceded by partial hepatectomy *(38, 39)*. Thus the properties of the physiologic system capable of removing O^6-methylguanine from DNA and the ability to reduce the levels of this abnormal purine in DNA before DNA replication may be of critical importance in determining susceptibility to the carcinogenic stimulus produced by treatment with N-nitroso compounds.

Repeated daily administration of DMN to rats by injection or feeding leads to a high incidence of liver cancer *(1, 20, 21)*. The present finding that O^6-methylguanine levels are not linearly related to dose of DMN has considerable practical significance if, as argued above, the formation of this purine is a critical step in the induction of cancer by this nitrosamine. If this phenomenon is maintained over a long period of time, and the experiments described in table 2 suggest that this may indeed be the case, it would imply that the average daily levels of this purine would be disproportionately greater after higher doses of DMN. The chances of initiating liver cancer may, therefore, also be disproportionately greater. Although a full dose response curve of liver tumor induction as a function of DMN intake is not available, there is some evidence that this does occur. DMN levels above 20 ppm in the diet led to liver cancer in more than 66% of the animals, whereas in rats fed diets containing 5 ppm the incidence was only 8% *(20, 21)*. These dietary levels correspond to daily intakes of about 0.5–2 mg/kg, respectively, which are well within the range in which O^6-methylguanine levels are not proportional to dosage. Although there is evidence with some carcinogens that repeated applications have an entirely cumulative effect, indicating that there is no recovery phase after each dose *(40)*, other experiments strongly suggest that it is possible to repair damage produced by DMN. The effects of two separate doses of DMN on renal tumor formation are not additive if a sufficiently long period is allowed between the two doses *(41)*.

A no-response level for DMN feeding has not been determined for liver cancer induction in the rat; even a diet containing only 2 ppm DMN led to 1 tumor in 37 treated rats *(21)*. The present data do not necessarily indicate the existence of a threshold dose of DMN below which no significant increase in tumor production might be expected, for it is not known what level of O^6-methylguanine must be present in the DNA in order to initiate tumor induction. However, these observations do suggest a mechanism by which the liver might be able to protect against the carcinogenic stimulus. At the lowest dose of DMN for which measurements were made, 0.25 mg/kg body weight, the amounts of O^6-methylguanine which were present at 4 and 24 hours after injection were about 1.0 and 0.25 moles/10^6 moles of guanine, respectively. From the value obtained for 7-methylguanine formation after this dose of DMN (85 moles/10^6 moles of the parent base) and the expected ratio of methylation at the O^6-:7-position of 0.11, we can calculate that the expected initial value of O^6-methylguanine should be 9.4 moles/10^6 moles of guanine. Thus about 90% of this appears to be lost in the first 4 hours and more than 97% in 24 hours. The high efficiency of this system may, therefore, be able to protect almost completely against the carcinogenic effects of very low doses. Conclusive proof of this requires a much better understanding of the underlying reactions responsible for the loss of O^6-methylguanine from DNA. Attempts to purify the enzymes involved and to characterize the reactions leading to the loss of O^6-alkylguanine from DNA are in progress, and activity has been detected in cellfree extracts from rat liver *(9, 36)*. If an inhibitor can be discovered that prevents the loss of O^6-methylguanine in vivo, it might be possible to confirm the role of the persistence of this product in DNA in carcinogenesis.

The data of table 2 indicate that, even after repeated daily doses of DMN, the level of O^6-methylguanine produced by 0.5 mg DMN/kg was less than 2% of that produced by a tenfold greater dose. It is of course quite possible that more prolonged treatment with the carcinogen would lead to alterations in these levels, particularly as the effect of long-term administration on the metabolic activation of DMN has not been studied. Also, under long-term treatment there may be changes in the physiologic system removing O^6-methylguanine from DNA. Although this system is more efficient after lower doses of DMN, the absolute amount of O^6-methylguanine lost from DNA is greater after the higher doses. It is possible that two processes are removing this product and that one becomes saturated at a much lower level than does the other. However, there are many other tenable hypotheses. For example, if alkylation after lower doses of the carcinogen occurs preferentially at certain regions of the DNA (perhaps those involved in transcription) which are not masked by chromatin proteins, it might be easier for the removal enzymes to excise the O^6-methylguanine. Recently, evidence has

been presented to suggest that both distribution and removal of DMN-induced methylated products in rat liver chromatin DNA are nonrandom *(42, 43)*. Alternatively, the removal process might be directly influenced by the high dose of the carcinogen, perhaps by inactivation of an essential enzyme component by alkylation.

The experiments showing that O^6-methylguanine is rapidly lost after the treatment with *N*-methyl-*N*-nitrosourea indicates that this process is not limited to DMN-treated rats but may be a general phenomenon occurring with all alkylating agents that give initial levels of O^6-alkylguanine less than 50 moles/10^6 moles of guanine. Very recently, Kleihues and Margison *(44)* reported studies that strongly support this proposition and are in general agreement with the other results in this paper. They studied the levels of labeled alkylated bases in the rat liver 6 hours after treatment with radioactive *N*-methyl-*N*-nitrosourea and found that pretreatment of the rats with a number of unlabeled alkylating carcinogens including *N*-methyl-*N*-nitrosourea, DMN, methyl methanesulfonate, and *N*-ethyl-*N*-nitrosourea led to an increase in the amount of labeled O^6-methylguanine present, but not of labeled 7-methylguanine. The simplest interpretation of these data is that the system responsible for removal of O^6-methylguanine is already overloaded by the unlabeled base produced in the prior treatment with the alkylating carcinogens and is thus unable to remove the labeled O^6-methylguanine as efficiently as in the animals that had not been pretreated.

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